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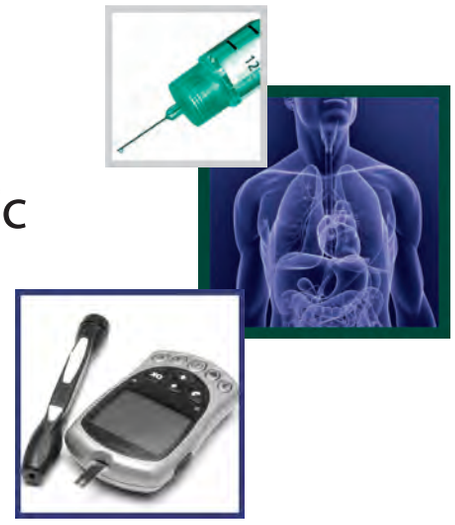

Viruses and Type 1 diabetes: a dynamic labile equilibrium

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Practice Points

- Epidemiological data suggest the increasing importance of environmental factors in Type 1 diabetes (T1D) pathogenesis.
- Histopathological features of early T1D suggest viruses to be prime environmental candidates.
- The hypothesis of enteroviral involvement in T1D was formulated in 1974 but the first proof of the association between enteroviridae and T1D came 40 years later, when virus protein 1-immunopositive cells were detected in multiple islets of 44 out of 72 young recent-onset T1D patients, compared with three islets from three out of 50 normal controls.
- The majority of the histopathological hallmarks of early diabetes pathogenesis could be explained by viral infection: insulitis, MHC I upregulation and interferon production.
- Enteroviruses have a strong pancreotropism and islets show strong expression of the coxsackie adenovirus receptor.
- Enterovirus can induce diabetes in animal models, but also protect from the development of T1D in other animal models.
- More systematic analyses are needed to address the role of enterovirus in T1D.
- These analyses can only be performed via a concise collaborative effort of scientists from various fields.
- A very promising approach in this regard is the recently founded Network of Pancreatic Organ Donors–Virus consortium.

SUMMARY Type 1 diabetes (T1D) results from the specific immune-mediated destruction of the insulin-producing β-cells of the pancreas. In genetically susceptible individuals, a still undetermined initiating 'hit' triggers a cascade of events that eventually leads to autoreactive CD8 T cells infiltrating the pancreatic islets and, subsequently, destroying them. There is increasing evidence that viruses, especially enteroviruses, are major environmental candidates; however, despite decades of investigation, we still lack certainty with regard to the causation of T1D. Moreover, studies in animal models of diabetes suggest a protective role of certain enteroviral infections upon diabetes contraction, making the quest for viral involvement in

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T1D even more difficult. Analyzing the foundation and the results of the most current work in the field, this article gives a brief overview of current knowledge, as well as providing an outlook for future directions.

There is robust evidence for the genetic basis of Type 1 diabetes (T1D), especially with regard to permissive HLA genotypes. However, the frequency of autoimmune diseases has increased considerably in recent decades, the annual increase in T1D incidence is currently estimated to be 3% [1]. This rising incidence, corroborated with a strong inhomogeneity in its geographical distribution, has to be attributed to environmental changes [2,3]. Moreover, the distribution of many autoimmune diseases forms a gradient between the northern and southern hemispheres [4], and the first generation of offspring from immigrants adopt the incidence rate of their new home country [2,5].

The hypothesis that viruses, mainly enteroviruses (EV), might be involved in T1D pathogenesis has a long history; however, we are still lacking a strong causal association between virus infection and T1D. Recent developments in analysis techniques, as well our access to organ libraries such as the Network of Pancreatic Organ Donors (nPOD) in the USA or the collection of Foulis in the UK offer unprecedented opportunities to study these correlations. In this article, we give a brief overview of current studies in the field, as well as providing an outlook for possible future research.

Methods

The information contained herein was gathered by means of a meta-analysis performed using the National Library of Medicine database. The search engines were Google, as well as the National Library of Medicine proprietary search engine PubMed. Furthermore, the abstracts from the Scientific Sessions of the American Diabetes Association, as well as abstracts from the European Association for the Study of Diabetes conference 2012 were used, where applicable.

Brief history

The first expression of the paradigm of EV causes of T1D was formulated in 1974 by Nerup and colleagues who suggested that, in genetically determined hosts, the immune response fails to eliminate an infecting virus (coxsackie virus B [CVB] 4) that, in turn, might infect the pancreatic β-cells and trigger an autoimmune response [6].

Almost four decades later, Dotta and colleagues demonstrated, using electron microscopy, the presence of VP1 capsid protein in β-cell specimens from three out of six T1D organ donors [7]. Richardson and colleagues demonstrated that VP1-immunopositive cells were detected in multiple islets of 44 out of 72 young recent-onset T1D patients, compared with a total of only three islets in three out of 50 neonatal and pediatric normal controls [8]. More recently, the same group found islets positive for VP1 in some β-cells in more than 60% of patients with recent-onset T1D, but in very few age-matched controls within the Foulis pancreas tissue collection [9]. In addition, unpublished studies from the nPOD-virus consortium [Oikarinen M & Hyöty H, Unpublished Data] suggest that a high proportion of the nPOD T1D collection, in which residual β-cells persist, also display evidence of EV infection (as judged by immunostaining for viral protein). This is not the case for age-matched controls.

The epidemiological studies have not been as conclusive thus far. In a recent meta-analysis, Yeung and colleagues found a strong association between EV infection (based on detection with molecular methods) and T1D [10], in contrast with older analyses that suggested the opposite [11]. However, a marked heterogeneity in study design and methods renders these results, while suggestive, insufficient to prove the causal role of EV infection in T1D. For instance, MIDIA and babydiet reported no significant association between EV traces in stool samples and islet autoimmunity [12,13], and the DAISY study found no significant association with islet autoimmunity neither with RNA analyses in serum, saliva or rectal swab samples [14] nor serologically [Rewers M, Unpublished Data]. Furthermore, in a recent study, Yeung and colleagues showed that children with islet autoimmunity have higher levels of multiple cytokines, consistent with an active inflammatory process in the prediabetic state, yet these findings were unrelated to coincident EV infection [15]. However, the DIPP study found a close temporal association between the appearance of the first diabetes-associated autoantibodies and EV

infections [16,17], and similar results were seen in the TRIGR study [18].

Recently, Oikarinen and colleagues reported small, yet significant, differences in the frequency of serum EV RNA during follow-up of 38 cases who progressed from islet autoimmunity to T1D versus matched controls [19]. The difference in the frequency of enterovirus RNA between case children and control children was highest during the 6-month period before the appearance of the first autoantibody. An accelerating effect of EV infections on progression from autoantibody positivity towards T1D was also seen in the older DiMe study [20,21] as well as in the more recent DAISY follow-up study [22].

Notably, a common finding in almost all longitudinal studies was that in the same individual, EV RNA can rarely be found continuously in stool samples for more than approximately 3 months, and for a much shorter time in serum samples [16,14]. It is estimated that EV RNA can be found in serum for 2 weeks at most; however, there is no conclusive evidence for this. This is an important confounding factor underlining that negativity for virus at diagnosis does not mean missing viral etiology but strengthens the hypothesis of a 'hit and run' scenario with multiple viral infections leading cumulatively to pathological effects upon β-cells. Another confounding factor is the seasonality of EV infections. In humans, data from prospective studies suggest a seasonal pattern in the appearance of autoantibodies that resembles the seasonality of EV infections [23]. However, the seasonal pattern observed in the onset of clinical T1D is rather modest and, on another note, EV herd immunity is low in countries with the highest incidence of T1D [24]. Furthermore, the increase in T1D incidence has been rather accompanied by the decrease in EV infections during recent decades [25–27]. One explanation could be that, in those countries, low EV herd immunity leads to children getting their first EV infection at a later age when protection by maternal antibodies have already ceased, therefore, the outcome of an infection can be more severe (i.e., spreading to the pancreas).

Notably, it has recently been demonstrated that gestational EV infections are associated with an increased risk for T1D in the offspring: EV-IgM in early pregnancy increased the risk for islet autoantibodies at delivery in nondiabetic mothers with HLA-DQ 2/2 or 2/X T1D risk genotypes [28].

Hallmarks of T1D pathogenesis with reference to viral infections

In recent years, the authors and others have performed extensive studies on the histopathological features of T1D within the nPOD cohort and, in spite of still restricted concludent information about the prediabetic period, a vast amount of histopathological features of T1D have been found that can be explained by viral infection.For instance:

- Insulitis, which is considered to be a hallmark of early T1D pathogenesis, was only found in two out of the three cases that presented at least three autoantibodies, but in none of the other 59 antibody-positive subjects or 62 matched controls [29]. Thus, if autoantibodies are present and insulitis is only observed in a few cases, we have to think of T1D as a relapsing–remitting disease. One of the possible explanations for this scenario is recurrent viral infection: either *de novo* infection or flare-up of a chronic infection, or viruses, which persist but do not permanently reside in the pancreas (e.g., *Herpesviridae*);

- At the time of diagnosis, the pattern of insulitis is not homogenous, as would be expected in the case of a stochastic development, but is lobular [30,31]. Again, one of the explanations for this phenomenon could be viral infections, as well as vascular or neuronal factors;

- The upregulation of MHC I on most islets, a phenomenon previously described by Foulis *et al.* [32], that can persist for years [31] and was also found to occur without a concurrent inflammatory infiltrate; defined as the presence of inflammatory cells [7,33]. One hypothesis able to concatenate these findings can be the virus-induced secretion of interferons [34,35]. The presence of IFN-α and MHC I upregulation are concomitant events in β-cells [32] and the ability of both IFN-α [32] and IFN-γ [34,36] to induce MHC I upregulation is well known. The persistence of EV in islet cells is associated with the chronic synthesis of IFN-α [35,37] and other cytokines [37] in human islets inoculated with CVB3 virus. Furthermore, CVB4, in the presence of

antibodies and through the specific viral receptor coxsackie adenovirus receptor, was shown to infect monocytes resulting in IFN-α synthesis [38]. In addition, in a mouse model of virus-induced diabetes [34], as well as in the nonobese diabetic (NOD) mouse model [39], the presence of IFN-γ was essential for the contraction of diabetes. However, despite strong evidence for the presence of viral protein within islets [7], we still lack conclusive proof of viral genomes in islets or β-cells;

- Viral infections can upregulate MHC I **(Figure 1)**, therefore, creating a fertile, inflammatory field that eventually leads to the unmasking of β-cell antigens with subsequent infiltration with $CD8^+$ cells and the killing of the β-cells through direct and indirect mechanisms [40];

EVs have especially been shown to have a strong pancreotropism: severe islet damage was demonstrated in fatal CVB infection cases [41], islets demonstrate strong expression of the coxsackie adenovirus receptor [35,42] and β-cells are permissive for EV *in vitro* [43].

Taken together, these observations suggest that T1D might evolve through a series of inflammatory 'hits' affecting certain areas of the pancreas in a relapsing–remitting fashion, and that viruses may well fit into this scenario.

Direct pathogenetic associations between EV infections & T1D

In spite of the fact that a direct causal link between EV infection and T1D contraction is still lacking in humans (except in rare cases of fulminant T1D where an association with EV infection was discussed in case reports [44]), there are several examples of EV-induced diabetes in animals: we have seen diabetes development upon infection with CVB [45] and encephalomyocarditis virus [46] in mice; with the Lyungan virus in voles [47]; Kilham virus in rats [48]; and with the bovine viral diarrhea virus in cattle [49].

Viral infection can protect from diabetes

Under certain circumstances, infection with CVB can either accelerate (in the lymphocytic choriomeningitis virus diabetes mouse model) or abrogate (in the NOD mouse model, when induced at an early timepoint) the development of T1D [50]. The author's studies suggest that the timing of the infection is, in the latter case, very important. Mechanistical explanations comprise the TGF-β augmentation of a bystander Treg cell population, as well as reduction in T-effector activity through virally induced PD-1L and TNF-α **(Figure 2)** [51,52]. When regarded from an evolutionary point of view, this hypothesis makes sense: the body tries to limit the damage provoked by its own immune system while fighting a viral infection by enhancing the Treg response. Transferring a small number of Treg cells, which would not normally suffice to provide protection from a NOD mouse that has previously been infected with CVB3 to an unmolested NOD mouse, will protect the latter from developing T1D – thus, the viral infection positively stimulates the Treg compartment [53]. These enhancing effects upon polyclonal Tregs are mainly elicited through TLR2 [53]. These observations could help explain the proverbial hygiene hypothesis or, to a lesser extent, the suggested protection from T1D development in mice by infection with certain helminthes [54].

Conclusion

Novel insights in the histopathology of T1D make a causal link to viral infections very probable. Viruses can initiate autoimmunity, promote it, and precipitate and abrogate the onset of disease; however, all these roles have yet to be confirmed in larger prospective studies.

There have been many discussions regarding whether there is a rationale for developing a vaccine for CVB. A separate analysis of probes from the Finnish DIPP study has shown a higher prevalence of viral CVB RNA in stool probes from children who developed T1D autoantibodies and, within these results, a higher occurrence of the subtype CVB1 [Hyöti H, Unpublished Data]. However, given the fact that there are more than 100 EV serotypes, more systematic studies are required with more uniform assays to prove a pathogenetic causality of such a strength that the development of a vaccine can ethically and financially be considered. In conclusion, prospective studies with high case numbers, a much more frequent sampling of various specimens and a highly standardized methodology are necessary to prove statistically significant associations between EV infections and the risk of islet autoimmunity or T1D. These analyses can only be performed via a concise collaborative

effort of scientists from various fields. A very promising approach in this regard is the recently founded nPOD-V consortium [101].

Future perspective

Access to more tissue specimens, the fast development of novel techniques and a new, collaborative effort will open new avenues in the research of the viral causes of T1D. However, we should also start thinking outside of the box and consider other viral pathogens than EV, which might be a better fit for the relapsing–remitting scenario and for the lobular fashion of the disease. One idea could be that the virus does not infect the endocrine pancreas, but instead infects other adjacent structures. Elegant studies by Dosch and colleagues have shown that the first structures to be involved in T1D pathogenesis might be sensoric neurons innervating the islet [55]. *Herpesviridae* are known to persist in neuronal structures and to occasionally descend to their target tissue; however, they can rarely be found there (hit and run theory).

The authors also encourage a closer look towards other environmental factors that affect viral infections, such as the intestinal microbiota. Recently, a small study including DIPP children who eventually progressed to T1D and controls, examined the intestinal microbiome in feces collected either before, at seroconversion or close to the diagnosis of overt T1D [56] and showed that, in comparison to controls, children who developed T1D developed a less diverse microbiome with preponderantly nonbutyrate-producing lactate-utilizing bacteria that prevented optimal mucin synthesis [57]. Since the microbiota are present at the sites used by viruses to gain entry to their host, they can potentially alter the course and outcome of infection.

The causal link between EV and T1D pathogenesis is yet to be found. It probably involves a complex interplay between viruses, β-cells, and the innate and adaptive immune systems in the given genotypical context of an individual. Finding these associations, although challenging, can be decisive in developing new preventive and therapeutic strategies to fight this disease.

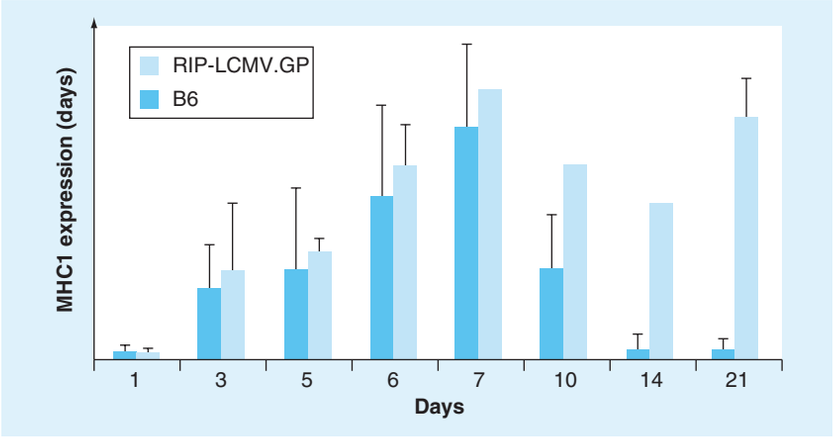

Figure 1. Time course of MHC class I induction in nontransgenic and rat insulin promoter-linked lymphocytic choriomeningitis virus glycoprotein-expressing transgenic mice after lymphocytic choriomeningitis virus infection.
Upregulation of MHC I occurs as early as 2 days postinfection (not shown) and reaches baseline levels in nontransgenic mice at approximately day 21 (no islet infiltration), whereas RIP-LCMV.GP transgenic mice continue to exhibit elevated MHC class I levels reflecting ongoing islet infiltration and destruction. At later timepoints, only very few islets were available in RIP-LCMV.GP mice, reflecting onset of Type 1 diabetes.
B6: Nontransgenic; RIP-LCMV.GP: Rat insulin promoter-linked lymphocytic choriomeningitis virus glycoprotein.
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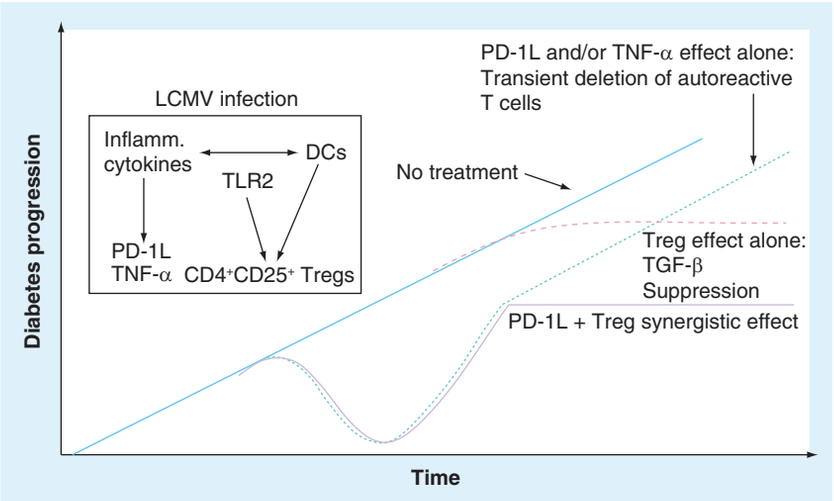

Figure 2. How viral infections stop Type 1 diabetes; TGF-β-mediated invigoration of a bystander Treg population, as well as downregulation of autoaggressive T cells through virally mediated induction of PD-1L and TNF-α.
DC: Dendritic cell; LCMV: Lymphocytic choriomeningitis virus.
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